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PRINCIPAL INVESTIGATOR: Gail L. Matters, Ph.D.

CONTRACTING ORGANIZATION: Pennsylvania State University
                         Hershey, Pennsylvania 17033-0850

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The overall objective of this project is to examine the role of the extracellular metalloproteases meprin A and B in breast cancer. The first step was to obtain full-length cDNAs encoding the human meprin α and β subunits and verify their expression in human cell cultures. A complete human meprin α cDNA was constructed and expressed in HEK293 cells, and it produced a functional meprin protein. This cDNA has been transfected into MCF-7 breast cancer cells under the control of a constitutive promoter. Stable MCF-7 transfectants are currently being screened for meprin expression. In addition, the human meprin α cDNA has been cloned into an inducible expression vector, and it is also being transfected into MCF-7 cells. A full-length meprin β cDNA is nearly complete and will be tested in HEK293 and MCF-7 cells. Fusion proteins containing the meprin A and B protease domains have been made and will be used as antigens for producing anti-meprin antibodies.

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FOREWORD

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INTRODUCTION

Progression of cancer cells from a non-invasive, non-metastatic phenotype to an invasive and metastatic phenotype is associated with the expression of a variety of cell surface proteases. Primary among these are the zinc metalloproteases known as matrixins, which include matrix metalloproteases such as collagenases and stromelysins. Matrixins can degrade extracellular matrix proteins, contributing to metastasis and angiogenesis. Another family of zinc metalloproteases are the 'astacins', which include the extracellular proteases meprin A and B. The expression of a novel form of meprin β mRNA only in cancer cells, and the secretion of meprin A protein by colon carcinomas has been previously documented. This indicates that these proteases play a role in cancer cell progression. The current project focuses on the expression of meprins A and B in breast cancer cells, and their potential role in tumorigenesis, invasion and metastasis.

SUMMARY

The major objective (Objective 1) for the research period May 1, 1998 – April 30, 1999 was to obtain and characterize full-length cDNAs encoding the human meprin α and β subunit proteins.

I have constructed a human meprin α cDNA and have shown that this cDNA produces a functional meprin A protein in cultured human cells (Objective 1, Task 1). The human meprin α cDNA was obtained by combining overlapping cDNA clones generated by RT-PCR encoding the 5' and 3' ends of the meprin α gene. The resulting full-length cDNA was subcloned into the mammalian expression vector pcDNA 3.1(+)(Invitrogen).

To test if the human meprin α cDNA produces an active recombinant protein, the meprin α cDNA was transfected into the human cell line HEK293. This is a readily transfectable kidney cell line used for routine production of recombinant mouse meprin A protein. If the human meprin α cDNA is expressed by these cells, it would show that the clone can produce a functional protein. HEK293 clones stably transfected with the human meprin α cDNA or with vector alone were selected and screened for secretion of meprin A protein into the media. A total of 21 stable cell lines were screened, of which 9 showed some level of human meprin A protein secretion. The three clones expressing the highest levels of meprin A protein were further characterized. Media from these cells was tested for proteolytic activity against a fluorogenic bradykinin analog (BK+) which is a known substrate for mouse meprin A. Media samples from the human meprin α transfectants

contained BK+ degrading activity, while media from vector only transfectants did not. This shows that the human meprin α cDNA clone I have obtained produces functional meprin A protein. The stable 293 cell transfectants of human meprin α will serve as positive controls for future studies of human meprin α expression in breast cancer cells (Objective 3).

Experiments to map the 5' and 3' ends of the meprin α transcript from MCF-7 breast cancer cells (Objective 1, Task 2) have been started. Thus far I have been unable to isolate RACE products from MCF-7 cell RNA, although controls using colon cancer cell RNA have been working. It is possible that the untranslated regions of the meprin α transcript from MCF-7 cells is altered in such as way that the RACE primers I have chosen will not anneal. Other RACE primers, including some within the coding region of the meprin α mRNA, will be used in these ongoing studies. I am continuing studies of the meprin β transcripts from MCF-7 and SK-BR-3 breast cancer cells. Previous experiments indicate that the 5' end of the meprin β transcript from cancer cells has a longer 5' UTR than the meprin β transcript from normal intestine. RT-PCR using a 5' primer containing sequences identified in colon cancer cells to be unique to the longer meprin β transcript show that this sequence is present in the meprin β transcripts from breast cancer cells. These data are included in a manuscript that has been accepted for publication in Molecular Carcinogenesis. I will continue the RACE analysis of the 5' end of meprin β transcripts to verify the transcription start site in MCF-7 and SK-BR-3 breast cancer cells.

Another continuing project is to obtain and express a full-length human meprin β cDNA (Objective 1, Task 1). Using RT-PCR, I have produced several full-length human β cDNAs and have cloned them into the mammalian expression vectors pcDNA3.1+ and pSG5. To test whether these cDNAs produce functional human meprin B protein, the clones were transfected into HEK293 cells. However, unlike the human meprin α cDNAs, the human meprin β cDNAs have not produced meprin protein. Sequence analysis of several clones has revealed three mutations in the β cDNA, presumably the result of PCR errors. These base changes are being corrected and the resulting cDNAs will again be tested for their expression in transfected 293 cells. If I am unable to show expression of the human meprin β cDNA, a rat meprin β cDNA will be substituted. Within the protease domain, the rat clone is highly homologous to the human clone, and their substrate specificities should be similar. I have successfully expressed the rat meprin β clone in 293 cells. The rat β protein is associated with the cell membrane, it can be trypsin activated to a proteolytically active form, and it has in vitro activity against

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known meprin β substrates such as the bioactive peptide gastrin (unpublished results). However, the rat β clone will be used only if no other options are available.

The next objective of this study is to determine where meprin A and B proteins are localized in breast cancer cells (Objective 2, Task 1), and if the proteins are intact and assembled into oligomers (Objective 2, Task 2). In order to address these questions, antibodies against the human meprin A and B subunits are needed. To create meprin specific antigens, I have cloned the protease domain of the meprin A and B subunits into the pMAL fusion protein vector (New England Biolabs). The protease domain fusion proteins have been expressed in E. coli but are insoluble. Once solubilized, the fusion proteins will be used to inoculate rabbits for the production of human meprin antibodies. I am also exploring the posibility of generating peptides unique to the meprin α or β subunits to use as antigens.

Because the human meprin α cDNA clone was successfully expressed in HEK293 cells, I have transfected this construct into MCF-7 breast cancer cells and selected stable transfectants for further analysis. Screening by RT-PCR, I have detected two transfectants that express the human meprin α transcript. These clones are being further screened for production of the meprin A protein. In addition, I have subcloned the human meprin α cDNA into an inducible TET-OFF expression vector (Clontech), and I will transfect this construct into MCF-7 cells as well.

KEY RESEARCH ACCOMPLISHMENTS:

- Cloning of a full-length human meprin α cDNA
- Expression of a human meprin α cDNA in HEK293 cells
- Transfection of human meprin α cDNA into MCF-7 cells
- Subcloning of human meprin α cDNA into a TET-inducible expression vector
- Synthesis of a fusion protein antigen for production of human meprin α and β antibodies
- Completing construction of a full-length human meprin β cDNA

REPORTABLE OUTCOMES:

<u>Book chapter</u>
Matters, G.L. and Bond, J.S. Meprin B: Transcriptional and posttranscriptional regulation of the meprin β metalloproteinase subunit in human and mouse cancer cells. APMIS 1999; 107:19-27.

Manuscript in press

Matters, G.L. and Bond, J.S. (1999) Expression and regulation of the human meprin β gene in human cancer cells. Molecular Carcinogenesis, in press.

Meprin B: Transcriptional and posttranscriptional regulation of the meprin β metalloproteinase subunit in human and mouse cancer cells

GAIL L. MATTERS and JUDITH S. BOND

Department of Biochemistry and Molecular Biology, The Pennsylvania State University College of Medicine, Hershey Pennsylvania, USA

Matters GL, Bond JS & Meprin B. Transcriptional and posttranscriptional regulation of the meprin β metalloproteinase subunit in human and mouse cancer cells. APMIS 1999;107:19–27.

A novel mRNA isoform encoding the cell surface metalloproteinase meprin β is expressed in mouse teratocarcinoma cells and in a variety of cultured human cancer cells. In both mouse and human cells, the cancer cell-specific mRNA isoform, referred to as β′, has an extended 5′ UTR as compared to the meprin β mRNA isoform expressed in normal kidney and intestinal epithelium. The work herein aimed to determine the molecular mechanisms for the expression of meprin β and β′ in normal and cancer cells, respectively. Analysis of the 5′ end of the mouse meprin β gene revealed that the unique sequences in the β and β′ mRNA isoforms are encoded by separate exons that are alternately spliced, and transcribed from independent promoters. By contrast, the human meprin β and β′ mRNAs have identical sequences except for 87 additional bases in the 5′ UTR sequence of β′, indicating that a single, mixed usage promoter directs expression of the isoforms. The region upstream of the human meprin β′ transcription start site contained elements with homology to the promoters of intestine-specific genes, interspersed with AP-1 and PEA3 elements; the latter were essential to meprin β′ promoter activity in cancer cells. Phorbol myristal acetate increased meprin β′ mRNA levels in cultured human colon cancer cells, providing further evidence that AP-1/PEA3 sites are actively involved in meprin β′ expression.

Key words: Meprin expression; protease; transcriptional regulation; mRNA isoforms.

Judith S. Bond, Department of Biochemistry and Molecular Biology, The Pennsylvania State College of Medicine, H171, Hershey PA 17033-0850, USA.

Meprin B is a cell surface protease of the 'astacin family' of metalloendopeptidases, and the 'metzincin' superfamily (1, 2). The enzyme has activity against proteins such as protein kinase A and gelatin, polypeptide hormones such as glucagon, and bioactive peptides such as gastrin and cholecystokinin. Meprin B is a homooligomer of meprin β subunits; the subunits are type I membrane proteins which form disulfide-linked dimers and noncovalently associated tetramers (3). The amino acid sequence of meprin β, deduced from mouse, rat, and human cDNAs (3, 4, GenBank Accession #X81333), indicates a multidomain protein containing a signal and prosequence, a protease/catalytic domain with an HEXXH zinc binding motif, 'interaction domains' (MAM, MATH, AM, and EGF-like), membrane-spanning and cytosolic domains (Fig. 1). Meprin subunits are normally expressed in the brush border membranes of renal proximal tubule cells and intestinal epithelial cells. One gene for the β subunit has been identified in the mouse and human genome; it has been localized on chromosome 18 in both genomes (5).

Recently mRNAs for meprin β with higher than normal molecular masses (referred to as meprin β′) were identified in mouse and human cancer cells (6). The presence of these isoforms indicated special mechanisms or types of regulation were involved in the expression of meprin subunits in cancer cells. Because meprin B is found at the surface of cancer cells, it may have a critical role in processes such as the
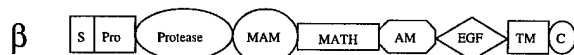
β S | Pro | Protease | MAM | MATH | AM | EGF | TM | C

Fig. 1. Domain structure of the meprin β subunit protein. S, signal peptide; Pro, propeptide; Protease/catalytic domain; MAM, meprin/A-5 protein/receptor protein tyrosine phosphatase μ domain; MATH, meprin and TRAF-homology domain; AM, aftermath domain; EGF, epidermal growth factor-like domain; TM, transmembrane domain, C, cytoplasmic domain.

activation of latent proteases, degradation of extracellular matrix proteins, or activation or degradation of growth factors. Therefore, it is important to investigate the mechanisms responsible for determining the expression and activation of these proteases. The present paper describes the expression of meprin β' mRNA in mouse and human cells, and differences between mouse and human isoforms.

MATERIALS AND METHODS

Cancer cell lines and culture conditions

Human colon adenocarcinoma cell line HT29–18C$_1$, osteosarcoma cell line U2 Os, and breast adenocarcinoma cell lines MCF-7 and SK-BR-3 were maintained in Dulbecco's modified Eagle's medium (Life Technologies) containing 10% fetal calf serum, 100 U/ml penicillin and 100 μg/ml streptomycin. The human pancreatic cancer cell line BxPC-3 was maintained in RPMI 1640 medium (Life Technologies) with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin and 100 μg/ml streptomycin. F9 and Nulli-SSC-1 cells were cultured as described previously (6).

5' and 3' analysis of cDNA ends

RACE (Rapid Amplification of cDNA Ends) was used to determine the 5' and 3' transcript termini for the human meprin β and β' mRNAs. For 5' RACE, 4 μg of RNA was reverse transcribed using an internal meprin β primer (5' AGGAGATCAGAGTCACTG 3') located approximately 700 bases downstream of the translation start site. RNA from the human colon cancer cell line HT29–18C1 was used for reverse transcription of the human meprin β' transcript, and human intestinal RNA (Clontech) was used for reverse transcription of the human meprin β transcript. Reversed transcribed cDNA was ligated to a cDNA adapter (5' Amplifinder RACE kit, Clontech) and PCR amplified using nested adapter primers and two nested meprin β primers (5' GCCACTGCCCTTGAACAC 3', and 5' GCATTGAGG-ATAACTCCC 3') located 300–400 bases downstream of the translation start site, within the protease domain of the protein. The meprin β primers were derived from the sequence of the human meprin β subunit cDNA, GenBank accession #X81333.

For 3' RACE, the same RNA samples were reverse transcribed using a modified oligo (dT) primer (5' GCGTCT-AGATCTCGAGCTCGT$_{16}$ 3'). PCR amplification used two nested meprin β primers from the 3' UTR (5'GTG-TGAAAAGAGAGGCTC 3' and 5' ACCCGAGACACC-ATAGTC 3') and a primer within the modified oligo (dT) (5' GCGTCTAGATCTCGAGCTCG 3'). RACE products were cloned into the TA vector (Invitrogen) and completely

sequenced using a Sequenase version 2.0 DNA sequencing kit (Amersham).

Analysis of Meprin β and β' mRNAs in human cancer cells

Reverse transcription PCR (RT-PCR) was used to detect the human meprin β mRNA in various cancer cell lines. Human fetal kidney RNA was purchased from Clontech. Total RNA was isolated from cultured cells using Tri-reagent (Molecular Research Center, Inc.) and 5 μg of total RNA was used in first strand cDNA synthesis reactions using an oligo(dT) primer. The amounts of cDNA in each sample was determined by PCR using primers for the constitutively expressed GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (5' CCACCCATGGCAAATTCC-ATGGCA 3' and 5' TCTAGACGGCAGGTCAGGTCC-ACC 3') gene. Equivalent amounts of template cDNA were then used for nested PCR using 5' primers specific to the meprin β' untranslated region (5' AGCATCAAGCTG-ACCTGC 3', and 5' GAAGTCAATTCAACCC TG 3') and 3' primers specific to the meprin protease domain (5' CAAAACTGCATGAGT CC 3', and 5' AGGAGATCA-GAGTCACTG 3'). PCR reactions were done at 50° to 54°C annealing temperatures for 30–35 cycles.

Cloning of human MEP1B promoter region

Genomic DNA encompassing the 5' UTR and upstream promoter region for the human *MEP1B* gene was cloned using a PromoterFinder DNA walking kit (Clontech). Regions of the *MEP1B* promoter were PCR amplified by employing primers specific to the 5' UTR common to both the human meprin β and β' mRNAs. The largest genomic fragment amplified, a 1.8 kb fragment, was cloned and restriction mapped. Approximately 700 bases of genomic DNA upstream of the β' transcript start site was completely sequenced. The *MEP1B* promoter was analyzed for areas of sequence similarity to other promoters using the Mat-Inspector program (release 2.1 with matrix table release 3.1) and the FastM program (release 1.0) from the Transfac database.

Construction of MEP1B promoter/luciferase reporter gene plasmids and transfection into human cancer cells

For functional examination of the *MEP1B* promoter, various portions of the promoter were PCR amplified and cloned into the luciferase reporter vector pGL3-Enhancer (Promega). Transient transfections of the human osteosarcoma cell line U2 Os in Opti-MEM serum-free media (Life Technologies) were done using DEAE-dextran. Log-phase cells were cotransfected with 5 μg of reporter gene plasmid and 5 μg of the pSV-β-galactosidase plasmid (Promega) in 500 μg/ml DEAE-dextran for 2 hours. Cells were shocked with 10% dimethyl sulphoxide in PBS for two minutes, washed twice with PBS, and transferred to Dulbecco's modified Eagle's medium. After three days cells were harvested and lysed in reporter gene lysis buffer (Promega). Cell extracts were assayed for β-galactosidase activity spectrophotometrically using ONPG (o-nitrophenyl-β-D-galactopyranoside) as a substrate. Luciferase activity was analyzed in duplicate using an Autolumat LB953 luminometer (EG&G Berthold) kindly provided by Dr. Kathryn LaNoue in the Dept. of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine. The luciferase activity, normalized per unit of β-galactosidase, of each promoter construct represents the mean of at least 12 independent transfections.

To create mutations in the AP-1 or PEA3 sites, or in both

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sites, the -83 construct of the *MEP1B* promoter was PCR amplified using primers with two base changes in either element, or one base change in each site for the double mutant (the altered bases are shown in bold). The *MEP1B* mutant primers are: AP-1: 5'-GCCAGGCACTTAGATCTGCAC-ACAGGAAA-3', PEA3: 5'-GCCAGGCACTTAGATCTT-GACACA**CC**AAAAAA-3', and the AP-1/PEA3 double mutant: 5'-GCCAGGCACTTAGATCTGGACACACGA-AA-3'. Mutations in these bases were previously shown to affect transcriptional activation of the collagenase promoter by these elements (9). As with the promoter deletions, the mutated promoter sequences were sequenced, subcloned upstream of the luciferase reporter gene, transiently cotransfected with the pSV-β-galactosidase plasmid into U2 Os cells, and assayed for reporter gene activity. For each mutant or wild-type construct, 5–6 independent transfections were assayed.

Induction of Meprin β' mRNA by phorbol ester

Log phase cultures of the human colon adenocarcinoma cell line HT29 18C₁ were transferred to serum-free media (Opti-MEM, Life Technologies) for 16 hours, and then treated with 50 or 100 ng/ml phorbol 12-myristate, 13-acetate (PMA, CalBiochem) for 8 hours. Cells were then extracted and total RNA isolated as described above. Total RNA (10 μg) was reverse transcribed and used as a template for PCR.

RESULTS

Structure of the mouse Meprin β' mRNA from teratocarcinoma cells

Northern blots of RNA from the mouse teratocarcinoma cell lines F9 and Nulli-SSC revealed that these cancer cell lines contained a 2.7 kb isoform of meprin β mRNA, designated as the meprin β' mRNA, that was larger than the 2.5 kb meprin β mRNA found in mouse kidney tissue (Fig. 2A). Retinoic acid treatment of the F9 and Nulli-SSC cells increased the amount of meprin β' mRNA. RT-PCR
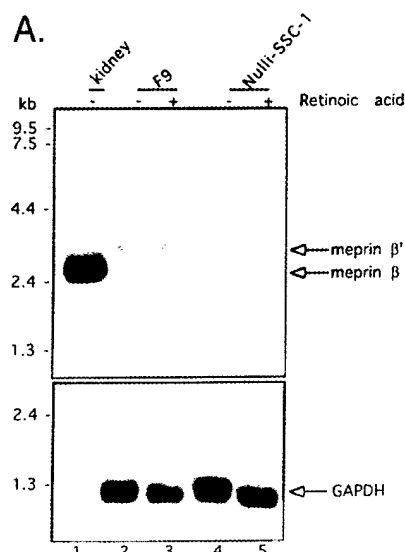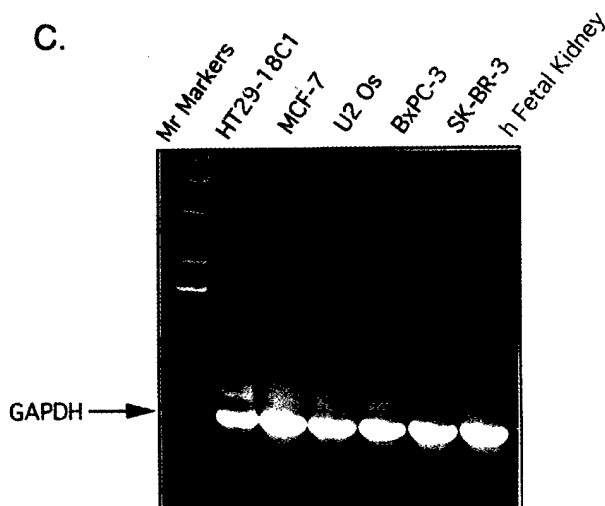

Fig. 2. **A.** Northern blot of mouse β and β' subunit and GAPDH mRNA from mouse kidney and embryonal carcinoma cells F9 and Nulli-SSC-1 cultured with or without retinoic acid. **B.** Reverse transcription PCR analysis of meprin β' mRNA, and **C.** glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA levels in the cultured human cancer cell lines. HT29–18C₁, colon adenocarcinoma; MCF-7, breast carcinoma; U2 Os, osteosarcoma; BxPC-3, pancreatic carcinoma; SK-BR-3, breast carcinoma; and h fetal kidney, human fetal kidney. Molecular weight (Mr) markers are a 1 kb DNA ladder (Life Technologies).

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A.

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HUMAN β'   ATTATTACAG CATCAAGCTG ACCTGCAATA TGGATCATTG AGATCAAAGG CTATAAATTA TAATGTTTGC CTTTAAAGAA   80
HUMAN β'   GTCAATTCAA CCCTGAATGT CATAGTTAGC TACTTTCAAC TGGAAGCTAC AACATGGATT TATGGAATCT GTCTTGGTTT   160
                      ||| ||||||||||| ||||||||||| ||||||||||| ||||||||||| ||||||||||| ||||||||||| |||||||||||
HUMAN β            CAA CCCTGAATGT CATAGTTAGC TACTTTCAAC TGGAAGCTAC AACATGGATT TATGGAATCT GTCTTGGTTT   73
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B.

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MOUSE β'                                                                                    GACATGTG   8
MOUSE β'   AGACGCTGGC TGGTCTCAAC AATTTGGCGA ACCAACGCCT GGGAAAGGCA GAGGACATTT GGAAGAAGCA CTGCTGGTTT   88
MOUSE β'   GGAGCTCCAT GGAAGAAGAA AAGAATTAAA GAAAATTCGT ACCGGAGAAG ATACTGCTAC CTGCTGAGAG GCCTGTCCTG   168
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HUMAN β'   ATTATTACAG CATCAAGCTG ACCTGCAATA TGGATCATTG AGATCAAAGG CTATAAATTA TAATGTTTGC CTTTAAAGAA   80
              |        |  |  |||||       |  ||   |        |   |  |   ||  |    ||| |   ||  |||
MOUSE β'   CACGTCATCA CCTGCAGCTG GTTCCAGGCT CCAAGGATGA ATCGGTGGGA ATGGACCTTC TCCCCTTTTC TTTATAAGGT   248
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HUMAN β'   GTCAATTCAA CCCTGAATGT CATAGTTAGC TACTTTCAAC TGGAAGCTAC AACATGGATT TATGGAATCT GTCTTGGTTT   160
              | | | |      ||      | | | |   | | |  ||  | |             |            |        | |
MOUSE β'   TTCCAGTTGA TGATGTTGCC TAGGGAGACA TGAATAGCAC AGCTGGACCA GCAAGCAGAA GCAGACACAG CTTCAAATGC   328
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HUMAN β'   CTGTTCTTGG ATGCTCTTCT CGTGATTTCT GGCTTGGCAA CTCCAGAAAA CTTT------ GATGTAGATGG CGGAATGGA   234
              | |         |            || || |        |                 || |||||||| ||||| ||
MOUSE β'   AGGATGAAGC TCCTGAAGGC TCCCAGGGAC GGGATGTACA TGATGACATT TGGAGTCAAA GACATAGATGG AGGAATTGA   408
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C.

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HUMAN β'   ATTATTACAG CATCAAGCTG ACCTGCAATA TGGATCATTG AGATCAAAGG CTATAAATTA TAATGTTTGC CTTTAAAGAA   80
HUMAN β'   GTCAATTCAA CCCTGAATGT CATAGTTAGC TACTTTCAAC TGGAAGCTAC AAC-ATGGAT TTATGGAATC TGTCTTGGTT   159
                      || | ||  |||||| | ||||||| || |    |||||    |  ||  ||| | |||  | |||||||||
MOUSE β            AGCTTGC AGCTTTCATC TGGAAGCCAC AGTAATGGAT GCCCGGCATC AGCCTTGGTT   57
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Fig. 3. Comparison of the nucleotide sequence of the 5′ end of meprin β′ mRNA isoforms. The human meprin β′ mRNA 5′ end is compared to the previously determined sequence for human β cDNA (GenBank Accession # X81333), to the 5′ end of the meprin β′ mRNA (B), which is expressed in teratocarcinoma cells, and to the mouse meprin β mRNA (C), which is expressed in normal kidney and intestinal cells. The upward arrow indicates the location of the splice junction between the 3′ exons common to both the mouse β and β′ mRNAs (in italics), and the alternatively spliced 5′ exons specific either to the mouse meprin β mRNA or to the mouse meprin β′ mRNA. The translation start site (ATG) for each mRNA is underlined.

analysis using primers that spanned the protein coding regions of the meprin β′ mRNA isoform showed there were no differences between the meprin β and meprin β′ mRNAs within the coding region of the protein. 3′ RACE (Rapid Analysis of cDNA Ends) analysis revealed that the 3′ ends of the meprin β and meprin β′ mRNAs were identical. However, the 5′ RACE results indicated the meprin β′ mRNA isoform was significantly longer than the meprin β isoform. Sequence analysis of the 5′ RACE products showed the meprin β′ mRNA contained 277 bases of additional 5′ noncoding sequence. The sequence of the signal peptide and a portion of the propeptide coding sequence also were significantly different from the meprin β mRNA. Although the sequence of the β′ mRNA signal peptide was very different from the β mRNA signal peptide, the in vitro translated product of the β′ mRNA was able to insert into canine microsomal membranes, indicating that the β′ mRNA signal peptide was functional.

Structure of the human Meprin β′ mRNA from cultured cancer cells

The initial observation of a unique mRNA encoding the human β meprin subunit protein was made in the human colon cancer cell line HT29–18C₁ (6). As in the mouse teratocarcinoma cells, no evidence for coexpression of both meprin β′ and meprin β mRNAs in human colon cancer cells was found. The human meprin β and β′mRNAs were characterized by 5′ and 3′ RACE analysis and by RT-PCR analysis of the internal mRNA regions. Primers spanning various sections of the meprin β coding region were used for RT-PCR amplification of mRNA from the colon cancer cell line HT29–18C₁. When compared to the sequence reported for the full-length human intestinal meprin β cDNA (GenBank accession #X81333), the sizes of the RT-PCR products from the colon cancer cells was identical. As with the mouse, 5′ and 3′ RACE was used to determine the beginning and endpoints of the meprin β and meprin β′

mRNAs. Comparison of the 3' RACE products from HT29–18C$_1$ cells and from normal intestine showed no sequence differences, indicating that the 3' terminus of the β' mRNA from the cancer cells was identical to that of the β mRNA from intact intestinal tissue. Comparison of the meprin β' 5' RACE product from HT29–18C$_1$ cells and the meprin β cDNA from normal intestine indicated there were sequence differences between the 5' termini of these mRNAs. Within the protein coding region the meprin β and meprin β' mRNAs were identical in sequence, however, the meprin β' mRNA from HT29–18C$_1$ cells contained an additional 87 bases of 5' UTR sequence (Fig. 3A). This is similar to the mouse meprin β' mRNA, except that the mouse β' mRNA contains an extra 277 bases of 5' UTR (6). When the extra sequence from the human meprin β' mRNA was compared to the 5' UTR of the mouse meprin β' mRNA, there was no significant sequence similarity (32% base identity) (Fig. 3B). In fact, the 5' UTR, signal peptide and portion of the propeptide coding sequence from the human mRNA was highly similar to the mouse β mRNA isoform (71% base identity) (Fig. 3C). The sequence differences between the human β' and mouse β' mRNAs indicated that there may be distinct mechanisms that generated these two cancer cell-specific mRNAs.

The initial observation of the meprin β' mRNA was made in only a human colon cancer cell line, however further investigation indicated that this isoform was also expressed in other types of cancer cells. The unique sequence of the β' mRNA 5' UTR allowed β'-specific PCR primers to be made. These primers were then used in RT-PCR screening for expression of the β' mRNA in a variety of human cancer cell lines. Meprin β' mRNA was detected in several different types of cancer cells, including breast adenocarcinoma cells MCF-7 and SK-BR-3, osteosarcoma cells U2 Os, and pancreatic ductal adenocarcinoma cells BxPC-3 (Fig. 2B). However, the β' mRNA was not present in RNA samples from normal fetal kidney. The presence of the meprin β' mRNA was not due solely to the presence of differentiated microvilli in the colon cancer cell line HT29–18C$_1$. The meprin β' mRNA was expressed in human cancer cell lines that were not of kidney or intestinal origin.

The presence of two mRNAs with different structure may indicate either that (1) the mRNAs are the products of two independent meprin β genes, perhaps the result of genetic rearrangements in the cancer cell lines, or (2) a single meprin β gene produces multiple mRNA. Southern analysis showed that the organization of *Mep-1*β in the genomic DNA from Nulli-SSC cells and the *MEP1B* gene from human colon cancer cells is identical to that from normal tissues (6). To investigate the mechanisms responsible for the generation of the multiple meprin β mRNA isoforms, the genomic DNA encoding the human *MEP1B* and mouse *Mep-1*β upstream region was cloned and analyzed.

Analysis of the genomic sequences at the 5' end of the human MEP1B *gene and the mouse* Mep-1β *gene*

To determine how the unique 5' ends of the meprin β' mRNAs are produced in mouse teratocarcinoma cells and in cultured human cancer cells, segments of the genomic DNA encoding the 5' end of the meprin β' mRNAs and upstream genomic sequences were cloned. For the mouse gene, this was accomplished by PCR amplification of a mouse YAC library clone containing the mouse *Mep-1*β gene using primers to various sections of the β' and β mRNA. For the human *MEP1B* gene, an anchored human genomic library (PromoterFinder library from Clontech) was PCR amplified using primers common to the 5' UTR from both the meprin β' and β mRNAs.

In the mouse, the 5' end of the meprin β mRNA isoform was encoded by two exons (designated 1 and 2), while the 5' end of the meprin β' mRNA was encoded by three exons (designated 1', 2', and 3'). The β' and β exons were arranged tandemly in the genomic DNA and were separated by a 1.6 kb intron (6, Jiang, Kumar, Milliron and Bond, unpublished data). Thus, the appearance of the novel meprin β' mRNA is the result of alternate splicing of the 5' exons of the mouse *Mep-1*β gene and the use of independent promoters to direct meprin β expression in normal kidney and intestine tissue and meprin β' mRNA expression in teratocarcinoma cells.

For the human *MEP1B* gene, 1.8 kb of genomic DNA upstream of the region common to both the meprin β' and β mRNAs were cloned and partially sequenced (Fig. 4A). Unlike the mouse genomic DNA, the 5' UTR of the human meprin β and β' mRNAs were not found on separate exons, but were continuous in the genomic DNA. A single exon contained all the DNA sequence for the 5' UTR of both the β' and β mRNAs. Because of this arrangement, the transcription start site and a potential TATA box used by the β mRNA are encoded by the same DNA sequence that encodes a portion of the β' mRNA 5' UTR. The human genomic DNA sequence indicated that mouse and human cells use completely different mechanisms for generating the meprin β and β' mRNAs. Rather than alternately spliced exons, the human β' and β mRNAs use different transcription start sites to generate different length 5' UTRs.

As yet it is not known if the human genomic DNA upstream of the human *MEP1B* gene contains DNA sequences that are homologous to the mouse β' exons. However, a possible reason why potential upstream exon sequences are not used in human cancer cells may relate to the sequence of the human

23

A.

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CGACCTCAGGTGGTCCACCCACCTTGACCTCCCAAACTGCTGGGATTACACATGTGAGCCACCATGGCCAAAAATTAGTATTCAAAAAATA
TTATCCTGCATCTTAAGTAAGTTGAATATGAAGCCATGGGAACTGAGAGAACAGGTTGTTTCTAAAGAGAAATTATCCCAAGAAAAGAAGG
AAAAAGTGAGAAAGAATTAAGAGATGGATTGGGATCATTATTTTATTAGAAATCAGTGACCATTCCTACTTTTATGACCCATGTCCCACCA
GTGTTTGCTTGATGGGAATTTTTAGGGGATGAGATACATTGCTATGTTACTACTCTATATGTGAATAACCGGTGTGCCTGATTTCTAGCAC
ATTAATGGATTTTAATAACTCAATAAATTATAGGCACTCAATAATTTTTATTAAATGGGTGAATGATAAAAAGCAGTGGACATAACTGGAT
ATATTAAAGCAGCATGGGGCTATATGAGAGGAGGAGTTTCATAAGATCAAGATTAAAAATTAATTTGATATATATATTTTTTGCTTAAGTG
GTGTTTACTAGTCTAAGACCAGTACACATACATTAGAGAATGTATTCATTAAGAAGTCTCAGCCAGGCACTTAATTGTTGACACAGGAAAA
AAAAAAGCCAACAAACCAACTCGCAGAGCGTGTGTTAAAAATACTGGACTTTCATCCCTTCATTGGAGGTATTATTACAGCATCAAGCTGAC
CTGCAATATGGATCATTGAGACCAAAGGCTATAAATTATAATGTTTGCCTTTAAAGAAGTCAATTCAACCCTGAATGTCATAGTTAGCTAC
TTTCAACTGGAAGCTACAACATG
                      Met
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B.
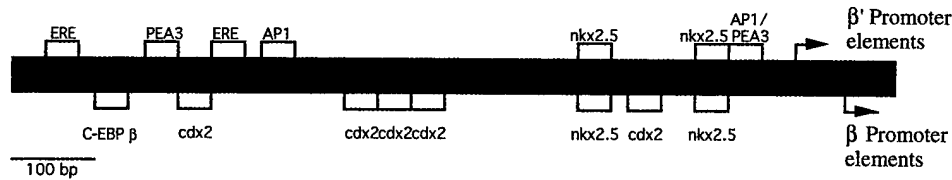
C.

Human SI (SIF1) $^{-55}$ GTGCAATAAAACTTTATGAGTA $^{-34}$
                     ||||||| | ||||
Human Meprin β $_{-58}$ CTGCAATATGGATCATTGAGAC $_{-37}$

Human SI (SIF2) $^{-86}$ AAGATTATTAAAC $^{-74}$ Human LPH $^{-155}$ TATTTTACAA $^{-146}$
                     ||||||| | ||||| |
Human Meprin β $_{-83}$ GGTATTATTACAG $_{-71}$ Human Meprin β $_{-160}$ GTGTTTACTA $_{-151}$

Human SI $^{-128}$ TACTTTCAG $^{-120}$ Human IFABP $^{-184}$ GAATTTCAG $^{-176}$
                     ||||||| || |||||
Human Meprin β $_{-106}$ GACTTTCAT $_{-98}$ Human Meprin β $_{-106}$ GACTTTCAT $_{-98}$

Fig. 4. **A.** Sequence of the human *MEP1B* promoter and 5'UTR. **B.** Location of potential upstream elements directing gene expression from the β or β' transcription start sites (indicated by the arrows). Elements of the β' promoter that may be involved in cancer cell-specific expression include estrogen-responsive elements (ERE), and PEA3 and AP1 transcription factor binding sites. Potential elements directing β-specific transcription include binding sites for the transcription factors C-EBP β and cdx2. Two potential binding sites for nkx 2.5 are also shown. **C.** Sequence similarities between the *MEP1B* promoter, and promoters of various human intestine-specific genes, including those encoding sucrase-isomaltase (SI), lactase phlorizin hydrolase (LPH), and intestinal fatty acid binding protein (IFABP). SIF1 and SIF2 are regions of the sucrase-isomaltase promoter defined by footprint analysis.

MEP1B gene at the splice junction where, in the mouse, the unique 5' exons and the exons common to both the β' and β mRNAs are joined. In humans, there is a 6 base deletion at this intron-exon junction as compared to the mouse sequence. This difference in the intron-exon junction may prohibit its use in splicing of potential upstream exons.

The use of closely spaced transcription start sites implies that the human meprin β and β' mRNAs employ the same segment of genomic DNA to encode the promoter elements that direct their transcription. To investigate this, approximately 700 bases of genomic DNA upstream of the β' mRNA start site were sequenced and compared to the promoters of other human genes. The *MEP1B* upstream region contained several areas with sequence similarity to promoter elements found upstream of both intestine-specific genes and genes that are highly expressed in cancer cells (Fig. 4B). Examples of potential intestine-

specific promoter elements included several binding sites for the caudal-related transcription factor cdx-2, which binds to the human sucrase-isomaltase promoter, and a binding site for the transcription factor C-EPBβ, which also is highly expressed in intestinal tissue. Direct comparisons of the *MEP1B* promoter to promoters of other genes expressed specifically in intestinal tissues showed several areas of sequence similarity (7, 8) (Fig. 4C). These included regions of the sucrase-isomaltase (SI) promoter that have been identified by footprinting as having proteins associated with them (SIF1, -37 to -58 in the *MEP1B* promoter, and SIF2, -71 to -83 in the *MEP1B* promoter). The region from -98 to -106 in the *MEP1B* promoter also had sequence similarity to portions of both the SI promoter and the intestinal fatty acid binding protein (IFABP) promoter.

Examples of potential cancer cell-specific promoter elements included AP-1 and PEA3 binding sites,

which are involved in the expression of other proteinase genes in cancer cells, and two potential estrogen-receptor binding sites, which may be involved in the expression of the β′ mRNA in breast cancer cells. Finally, two regions with high sequence similarity to the nkx2.5 transcription factor binding site were also detected. Nkx2.5 binding is generally associated with the transcriptional activation of cardiac-specific genes, however, there is evidence that this protein can also have repressor activity.

Functional analysis of the human MEP1B promoter

In order to test which upstream DNA elements affect transcription of the human meprin β′mRNA in cancer cells, various portions of the promoter were fused to a luciferase reporter gene and transiently transfected into U2 Os osteosarcoma cells. The activities of the largest promoter piece, containing 1800 bp of upstream sequence, was only about 2-fold higher than the vector alone, reflecting the fairly low level of β′ mRNA expression in cancer cells (Fig. 5A). However, the -1800 construct showed luciferase activity equal to the vector alone when transfected into human fetal kidney 293 cells, which do not express the meprin β′ or β mRNA (data not shown). Therefore although the level of promoter activity in cancer cells is low, it reflects the in vivo activity of this promoter. Promoter deletions containing 645, 461 and 83 bp of the *MEP1B* promoter were similar in activity to the 1800 bp construct, while a construct containing only 37 bp of the promoter, including the putative TATA box, was not different from the vector only. This indicated that the promoter region from

−37 to −83 bp contained an element(s) important for meprin β′ mRNA expression. Within this DNA region is a 12 bp element which resembles an overlapping AP-1/PEA3 binding site. Both AP-1 and PEA3 binding sites have been detected in the promoters of the MMP-1, MMP-3, MMP-7 and uPA genes, and have been shown to be critical for the expression of these genes in cancer cells (9, 10).

To further investigate the role of these sequences in meprin β′ mRNA expression, 2 bp mutations which will eliminate transcription factor binding to these elements, were made in either the AP-1 or PEA3 binding sites, or in both sites simultaneously. As before, the mutant promoter sequences were fused to a reporter gene and transfected into U2 Os cells. Mutations in either the AP-1 or PEA3 sites alone had no affect on reporter gene activity, however when both sites were altered, the promoter activity decreased to levels equal to the vector only constructs (Fig. 5B). This indicated that the two promoter elements could function independently in cancer cells, but that at least one of the elements was required for optimum promoter activity.

Phorbol esters increase Meprin β′ mRNA expression in colon cancer cells

Because the AP-1 and PEA3 sequence elements are important to the expression of the meprin β′ mRNA in cancer cells, this indicated that tumor promoters such as the phorbol ester PMA may affect levels of β′ mRNA. To test this, cultures of the human colon cancer cell line HT29–18C$_1$ were placed in serum-free media overnight, and then treated for 8 h with 50 or
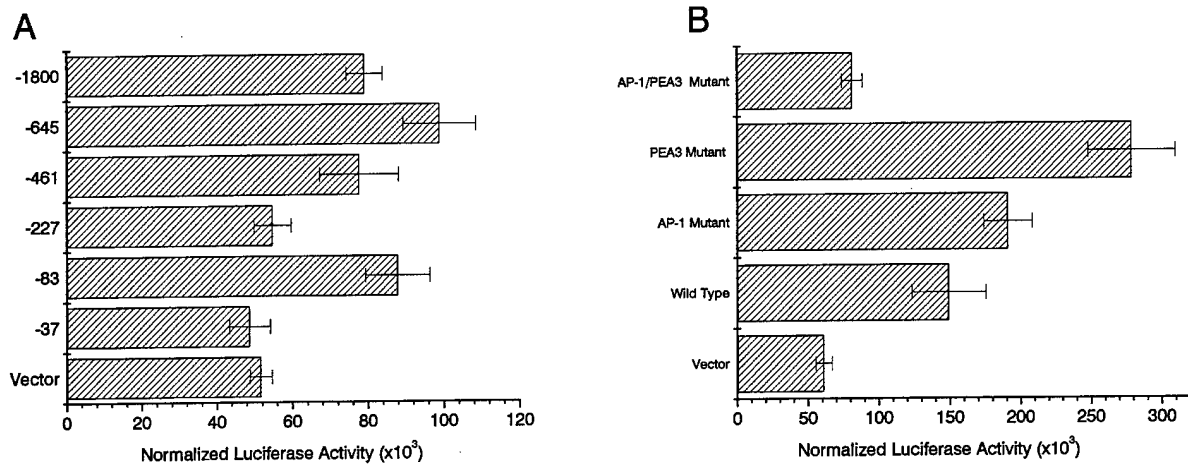

Fig. 5. **A.** Luciferase reporter gene activity, normalized to β-galactosidase activity, of the human *MEP1B* promoter constructs transiently transfected into U2 Os osteosarcoma cells. Vector represents the pGL3-Enhancer vector alone, while -37, -83, -227, -461, -645 and -1800 represent *MEP1B* genomic sequences upstream of the human meprin β′ transcription start site. Bars represent the standard error of the mean of at least 12 independent transfections. **B.** Luciferase reporter gene activity of the AP-1 or PEA3 sites with mutations introduced in one or in both sites simultaneously, of the *MEP1B* promoter. Constructs were transiently transfected into U2 Os cells. Bars represent the standard error of the mean of 5–6 independent transfections.
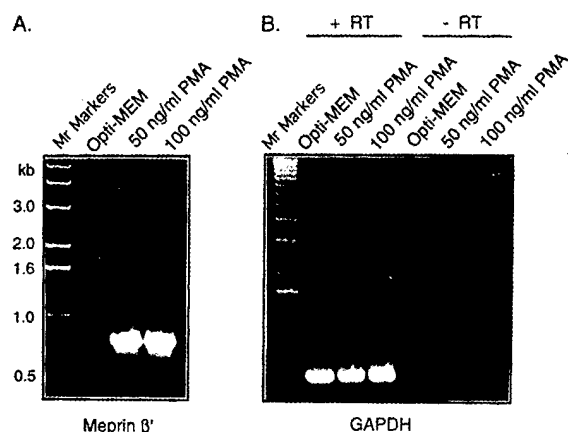
25

A.

Meprin β'

B.

GAPDH

Fig. 6. Induction of the human meprin β' mRNA by the phorbol ester PMA. Cultures of the human colon adenocarcinoma cell line HT29–18C₁ were transferred to serum-free media (Opti-MEM) with or without PMA as described in Materials and Methods. (A) RNA was reverse transcribed and used for PCR employing meprin β'-specific 5' primers. (B) As a control, reverse transcription reactions were done with or without reverse transcriptase, to eliminate the possiblity of genomic DNA contamination in the RNA sample, and PCR was done using GAPDH primers to show equal amounts of cDNA were present in each RT reaction.

100 ng/ml PMA. Total RNA from treated and untreated cells was analyzed for β' mRNA expression by RT-PCR. Incubation in serum-free media decreased the expression of the β' mRNA, however PMA treatments significantly increased β' mRNA levels (Fig. 6A). In contrast, PMA did not alter the expression of the constitutively expressed GAPDH gene (Fig. 6B), nor was genomic DNA present in the RT samples (-RT controls).

CONCLUSIONS

While both mouse and human cancer cell lines exhibit a novel isoform of the meprin β mRNA which is larger at the 5' end than the β mRNA found in normal tissues, the mouse and human meprin β' mRNAs are generated by distinct mechanisms. The mouse β and β' mRNAs encode proteins with different signal and propeptide regions, although the structure of the mature, activated proteins encoded by the β' and β mRNAs would be the same. These two mRNAs are generated by posttranscriptional mRNA modification through differential splicing of three exons located upstream of the exons for the mouse β mRNA and by transcription from independent promoters.

By contrast, the human β and β' mRNAs differ only in their 5' UTR and produce identical latent or activated proteins. The human β'-specific sequences are immediately upstream of the 5' end of the β-spe-

cific sequences with no intervening intron. Although the human β' and β mRNAs initiate at different transcription start sites, they use the same genomic DNA region as a promoter. DNA elements that contribute to the transcriptional regulation of the human meprin β' mRNA in cancer cells have been identified. A mixed promoter which contains sequence elements directing expression both in normal tissues and in cancer cells is also used by the human cathepsin D gene (11). Therefore, the mouse meprin β and β' mRNAs are produced by transcriptional and post-transcriptional mechanisms, while the human β and β' mRNAs differ only in the transcription start site used, and are not generated by posttranscriptional modifications.

Human meprin β' mRNA is expressed in a variety of cancer cell lines, not only in cancer cells which contain brush border membranes, such as the HT 29–18C₁ colon cancer cells. The human *MEP1B* promoter contains binding sites for AP-1 and PEA3 transcription factors, and β' mRNA levels in colon cancer cells are increased by treatment with the phorbol ester PMA. The presence of AP-1 and PEA3 binding sites and inducibility by phorbol esters are also characteristics of other proteinase genes, such as several of the matrix metalloproteases (MMPs), that are active in cancer cell lines (9, 10). Although the function of many MMPs in cancer cells has been well defined, the precise role of meprin B in cancer cells is still unknown. Meprin B has been shown to cleave the regulatory subunit of protein kinase A, as well as small biologically active peptides such as gastrin, cholecystokinin and glucagon (12, 13, Matters and Bond, unpublished data). The presence of meprin B on the cell surface places this protease in a position to regulate the activity of these peptides and potentially affect the growth of cancer cells.

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Expression and Regulation of the Meprin β Gene in Human Cancer Cells

Gail L. Matters and Judith S. Bond*

Department of Biochemistry and Molecular Biology, The Pennsylvania State University College of Medicine, The Milton S. Hershey Medical Center, Hershey, Pennsylvania

A novel mRNA isoform (meprin β') of the cell-surface protease subunit meprin β was previously identified in human colon cancer cells. The study reported here revealed that this mRNA isoform was identical within the protein coding region and at the 3' end to the β isoform of normal intestine but that it contained an extended 5' untranslated region. Meprin β' mRNA was expressed in the human breast cancer cell lines MCF-7 and SK-BR-3, in the human osteosarcoma cell line U2 Os, and in the human pancreatic cancer cell line BxPC-3. Meprin β mRNA, but not β' mRNA, was expressed in human fetal kidney cells. We cloned and sequenced genomic DNA encoding portions of the promoter region of the meprin β gene. The unique sequences present in the β' mRNA were present in the human genomic DNA immediately upstream of the transcription start site for the β mRNA. The human meprin promoter sequence was searched for potential transcription-factor binding sites, and putative activator protein-1, polyoma enhancer activator 3 (PEA3), SCAAT enhance-binding protein beta, and estrogen-receptor binding sites were identified along with binding sites for the intestine-specific cdx-2 transcription factor. The activity of meprin promoter/luciferase reporter gene constructs transfected into U2 Os cells was highest with constructs containing 83 and 639 bp of promoter DNA. These regions of the promoter each contain a putative PEA3 element. Treatment of the human colon adenocarcinoma cell line HT29-18C$_1$ with 50 or 100 ng/mL phorbol myristal acetate for 8 h increased meprin β' mRNA levels. Likewise, U2 Os cells transfected with the -639/luciferase or -1800/luciferase constructs showed a phorbol myristal acetate–inducible increase in reporter gene activity, indicating that the PEA3 element within the -639 construct or other elements further upstream respond to phorbol ester.

Key words: mRNA isoforms; cell-surface proteinase; metalloprotease.

INTRODUCTION

Expression of extracellular proteases has been linked to tumor progression and invasiveness in a variety of cancers. It has been proposed that the concerted action of multiple cell-surface and secreted proteases, many of which act to directly degrade extracellular matrix components or activate latent proteases and growth factors, leads to remodeling of the extracellular matrix by cancer cells. There are numerous examples of increased expression of proteinases in human cancer cell lines and in tumor tissues. These include metalloproteinases such as the matrix metalloproteases (MMPs) and adamalysins (reprolysins), serine proteases such as urokinajge plasminogen activator, and cysteine or aspartic proteases such as cathepsins B and D [1–4]. In many instances, cancer cells express proteases that are not expressed in the corresponding normal tissue, such as pepsinogen C in breast cancer cells [5], or secrete proteases that are normally intracellular enzymes, such as cathepsins B and D [6]. Finally, cancer cells produce novel enzymes with proteolytic activity [7,8].

Increased activity of extracellular proteases in cancer cells often is the result of transcriptional activation of the genes encoding these proteins [9]. MMPs such as MMP-2 (gelatinase A) and MMP-7 (matrilysin) are transcriptionally induced by growth factors and by phorbol esters [10,11]. The promoters for many proteases expressed in

cancer cells contain the transcription-factor binding sites activator protein-1 (AP-1) and polyoma enhancer activator 3 (PEA3). AP-1 sites bind a heterodimeric transcription factor composed of the fos and jun oncoproteins, whereas PEA3 sites bind the c-ets family of transcription factors [12]. Levels of fos, jun, and c-ets–type transcription factors increase during tumorigenesis and in response to tumor promoters, and these factors can act cooperatively to induce transcription of MMP and serine protease genes in cancer cells [13–16].

Meprin β (EC 3.4.24.63) is a homo-oligomeric metalloendopeptidase composed of meprin β subunits and localized to the brush-border membranes of kidney and intestine [17,18]. A member of the metzincin protease

superfamily, the meprin β subunit shares active-site sequence and structural similarities with MMPs and adamalysins [19]. Meprin β subunits are encoded by a single gene, *MEP1B*, which has been mapped to the human chromosomal region 18q12.2–18q12.3 [20].

A novel form of the meprin subunit mRNA, the β' mRNA, was recently identified in the human colon adenocarcinoma cell line HT29-18C$_1$ [21]. Based on northern blot analysis, the meprin β' mRNA of the HT29-18C cell line was larger than the meprin β mRNA from normal intestine, and there was no evidence of the expression of the kidney or intestinal meprin β mRNA isoform in HT29-18C$_1$ cells. In fact, human meprin β expression has only been detected in kidney and intestine tissues; to date, it has not been found to be expressed in any cultured cell line.

Two forms of the meprin β mRNA have also been identified in mouse cells and tissues. As in humans, the mouse β mRNA isoform is expressed only in kidney and intestinal tissues, whereas the larger meprin β' isoform has thus far been found only in the embryonal carcinoma cell lines F9 and Nulli-SSC [21]. The mouse β and β' mRNAs contain very different sequences in their 5' untranslated regions (UTRs) and in their signal-peptide and propeptide coding regions. Sequencing of the mouse meprin β genomic DNA revealed that the different 5' regions of the β and β' mRNAs were encoded by separate exons that are alternatively spliced to generate the two mRNA isoforms [21] (Jiang, Kumar, Milliron, and Bonds, manuscript in preparation). The two exons encoding the β-specific regions of the mRNA and the three exons encoding the β'-specific regions of the mRNA are separated by 1.6 kb of genomic DNA, indicating that the mouse meprin β and β' mRNAs are transcribed from independent promoters. There is no evidence that the meprin β and β' mRNAs are coexpressed in any mouse or human tissue.

In this study, we asked whether (i) human and mouse meprin β' mRNAs are similar in sequence and, hence, derived by similar mechanisms; (ii) expression of the hyman β' mRNA is limited to colon cancer cells or is associated with a number of different human cancer cell lines; and (iii) there are sequence elements in the human *MEP1B* promoter that direct transcription of the β' mRNA only in cancer cells and the meprin β mRNA only in kidney and intestinal tissues.

MATERIALS AND METHODS

Human Cancer Cell Lines and Culture Conditions

The human colon adenocarcinoma cell line HT29-18C$_1$, osteosarcoma cell line U2 Os, and breast adenocarcinoma cell lines MCF-7 and SK-BR-3 were maintained in Dulbecco's modified Eagle's medium (Life Technologies Inc., Rockville, MD) containing 10% fetal calf serum, 100 U/mL penicillin, and 100 µg/mL streptomycin. The human pancreatic cancer cell line BxPC-3 was maintained in RPMI 1640 medium (Life Technologies Inc.)

with 10% fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin.

5' and 3' Analysis of cDNA Ends

Rapid amplification of cDNA ends (RACE) was used to determine the 5' and 3' transcript termini for the human meprin β and β' mRNAs. For 5' RACE, 4 µg of total RNA was reverse transcribed by using an internal meprin β primer (5'-AGGAGATCAGAGTCACTG-3') located approximately 700 bases downstream of the translation start site. RNA from HT29-18C1$_1$ was used for reverse transcription (RT) of the human meprin β' transcript, and human intestinal RNA (Clontech, Palo Alto, CA) was used for RT of the human meprin β transcript. Reversed-transcribed cDNA was ligated to a cDNA adapter (5' Amplifinder RACE kit; Clontech) and amplified by polymerase chain reaction (PCR) with nested adapter primers and two nested meprin β primers (5'-GCCACTGCCCTTGAACAC-3' and 5'-GCATTGAGGA-TAACTCCC-3') located 300–400 bases downstream of the translation start site, within the protease domain of the protein. The meprin β primers were derived from the sequence of the human meprin β subunit cDNA, (GenBank accession #X81333).

For 3' RACE, the same RNA samples were reverse-transcribed with a modified oligo (dT) primer (5'-GCG-TCTAGATCTCGAGCTCGT$_{16}$-3'). The PCR amplification used two nested meprin β primers from the 3' UTR (5'-GTGTGAAAAGAGAGGCTC-3' and 5'-ACCCGAGA-CACCATAGTC-3') and a primer within the modified oligo (dT) primer (5'-GCGTCTAGATCTCGAGCTCG-3'). The RACE products were cloned into the TA vector (Invitrogen Co., San Diego, CA) and completely sequenced with a Sequenase version 2.0 DNA sequencing kit (Amersham Corp., Arlington Heights, IL).

Analysis of Meprin β and β' mRNAs in Human Cancer Cells

RT-PCR was used to detect the human meprin β mRNA in various cancer cell lines. Human fetal kidney RNA was purchased from Clontech. Total RNA was isolated from cultured cells with Tri-reagent (Molecular Research Center, Inc.) and 5 µg of total RNA was used in first-strand cDNA synthesis reactions with an oligo (dT) primer. The amount of cDNA in each sample was determined by PCR with primers for the constitutively expressed hypoxanthine phosphoribosyl transferase (*HPRT*) (5'-CCAAAGATGGTCAAGGTCGC-3' and 5'-CTGCTGA-CAAAGATTCACTGG-3') or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (5'-CCAC-CCATGGCAAATTC-CATGGCA-3' and 5'-TCTAGA-CGGCAGGTCAGGTC-CACC-3') genes. Equivalent amounts of template cDNA were then used for nested PCR with 5' primers specific to the meprin β' UTR (5'-AGCATCAAGCTGACCTGC-3' and 5'-GAAGTCAAT-TCAACCCTG-3') and 3' primers specific to the meprin protease domain (5'-CAAAACTG-CATGAGTCC-3' and 5'-AGGAGATCAGAGTCACTG-3'). PCR were performed at annealing temperatures of 50°–54°C for 35 cycles. As an additional PCR control, primers

to the human *villin* gene (5'-CCCACCTTCACAGGCTGG-3' and 5'-TTTGCCACAGAAGTTTGTGC-3'), which is highly expressed in intestinal and kidney epithelial cells, were also used.

Cloning of Human *MEP1B* Promoter Region

Genomic DNA encompassing the 5' UTR and upstream promoter region of the human *MEP1B* gene was cloned with a PromoterFinder DNA walking kit (Clontech). Regions of the *MEP1B* promoter were PCR amplified by using primers specific to the 5' UTR common to both the human meprin β and β' mRNAs. The largest genomic fragment amplified, a 1.8-kb fragment, was cloned and restriction mapped. Approximately 700 bases of genomic DNA upstream of the β' transcript start site were completely sequenced. By using the MatInspector program (Release 2.1; with matrix table, Release 3.1) and the FastM program (Release 1.0) both from the Transfac database [22], the *MEP1B* promoter was analyzed to determine areas of sequence similarity between it and other promoters.

Construction of *MEP1B* Promoter/Luciferase Reporter-Gene Plasmids and Transfection into Human Cancer Cells

For functional examination of the *MEP1B* promoter, various portions of the promoter were PCR amplified and cloned into the HindIII site of the luciferase reporter vector pGL3-Enhancer (Promega Corp., Madison, WI), and the promoters were completely sequenced. For all promoter constructs, the 3'-amplification primer was 5'-GCTGTAÅTAAAGCTTCCAATGAAGGG-3'. The 5' amplification primers were as follows: -37, 5'-CCAACTCGCA-GAGATCTGTTA-3'; -83, 5'-GCCAGGCACTTAGATCT-TGACACAGG-3'; -227, 5'-GCATGGGGCTAGCTGAG-AGGAGGAG-3'; -461, 5'-GAAA-ATCAGTGAGCTCTCC-TAC-3'; -639, 5'-ATACCATG-TGAGCCACCCGGGC-CAA-3'; and -1800, 5'-CCCCA-GTGTAAGATCTTGGC-3'. The 3' ends of these constructs end at the β' transcription start site, and the 5' ends of the constructs are indicated in Figure 1. Transient transfections of the human osteosarcoma cell line U2 Os in Opti-MEM serum-free medium (Life Technologies) were performed with diethylaminoethyl-dextran. Log-phase cells were cotrans-

Figure 1. DNA sequence of the human *MEP1B* genomic upstream region and 5' UTR for the meprin β and β' mRNA isoforms and identification of potential promoter elements. "β'" indicates the transcription start site for the β' mRNA isoform, as determined by 5' RACE. "^β1" indicates the 5' end of the human meprin β cDNA, and "^β2" indicates the 5' end of the human intestinal mRNA isoform, as determined by 5' RACE. The vertical lines with numbers define the 5' ends of the promoter used in the luciferase reporter gene constructs. Potential TATA-factor binding sites are underlined. Putative transcription factor binding sites are shown in bold print. All numbering is relative to the meprin β' mRNA transcription start site.

fected with 5 μg of reporter gene plasmid and 5 μg of the pSV-β-galactosidase plasmid (Promega Corp.) in 500 μg/mL diethylaminoethyl-dextran for 2 h. The cells were shocked with 10% dimethyl sulphoxide in phosphate-buffered saline for 2 min, washed twice with phosphate-buffered saline, and transferred to Dulbecco's modified Eagle's medium. After 72 h, the cells were harvested and lysed in reporter-gene lysis buffer (Promega Corp.). Cell extracts were assayed for β-galactosidase activity spectro-photometrically by using o-nitrophenyl-β-D-galactopyra-noside as a substrate. Luciferase activity was analyzed in duplicate using an Autolumat LB953 luminometer (EG&G Berthold) kindly provided by Dr. Kathryn LaNoue (Department of Cellular and Molecular Physiology, The Pennsylvania State University College of Medicine). The luciferase activity, normalized per unit of β-galactosidase, of each promoter construct represents the mean of at least 12 independent transfections.

Induction of Meprin β′ mRNA by Phorbol Ester

Log-phase cultures of the human colon adenocarci-noma cell line HT29 18C$_1$ were transferred to serum-free medium (Opti-MEM; Life Technologies) for 16 h and then treated with 50 or 100 ng/mL phorbol 12-myristate, 13-acetate (PMA; CalBiochem Novobiochem Corp., San Diego, CA) for 8 h. The cells were then extracted, and total RNA was isolated as decribed above. Total RNA (10 μg) was reverse transcribed and used as a template for PCR.

Effect of Phorbol Ester on *MEP1B* Promoter Activity

U2 Os cells were transfected with *MEP1B* promoter/luciferase plasmids as described above. Twenty-four hours after transfection, the cells were transferred to Opti-MEM medium and 100 ng/mL PMA was added. After an additional 24 h, the cells were harvested and assayed for reporter gene activity.

RESULTS

Differences at the 5′ Ends of Human Meprin β′ and β mRNAs

Previous results indicated that the meprin β′ mRNA found in human colon cancer cells is larger than the meprin β mRNA found in normal intestine and that no meprin β mRNA is detected in cancer cells [21]. The larger meprin β′ mRNA could be caused by additional sequences at the 5′ or 3′ end or to extra sequences within the coding region of the β′ mRNA. RT-PCR using primers to a variety of sequences within the coding region of the meprin β′ mRNA showed no difference in the sizes of the RT-PCR products from HT29-18C$_1$ cells and the size of RT-PCR products from meprin β normal intestine (data not shown). To determine if the β′ and β mRNA size difference was due to extensions at either end of the meprin β′ mRNA, RACE analysis of the 5′ and 3′ ends of the human meprin β′ and β mRNAs was performed. RACE products were amplified with RNA from HT29-18C$_1$ cells, which contains only the β′ mRNA,

and with RNA from human fetal intestine, which contains only the β mRNA. In all instances, a single RACE product was obtained. Sequence analysis of the 3′ RACE product from the colon cancer cells and from fetal intestine revealed that the 3′ termini of the meprin β and β′ mRNAs were identical. Our results did differ from the sequence of the meprin β subunit cDNA from human intestine reported in GenBank (accession #X81333). In both the β and β′ 3′ RACE products, there were three additional in-frame bases, which encode a glutamine residue. Insertion of this residue between amino acids 697 and 698 makes the COOH-terminal amino acids of the human protein N-Q-H-A-F and makes this human meprin β sequence identical to the meprin β sequence from the mouse and rat.

Comparison of the 5′ RACE product from HT29-18C$_1$ cells and from normal intestine did reveal sequence differences. The intestinal meprin β 5′ RACE product was identical in sequence to the reported human meprin β cDNA with the exception of being 15 bases shorter than the cDNA sequence. The meprin β′ 5′ RACE product from the colon cancer cells contained all the sequences present in the intestinal 5′ RACE product (Figure 2A) as well as 87 additional bases of 5′ untranslated sequence beyond the end point of the cDNA. Thus, the cancer cell β′ mRNA contains a 133-base 5′ UTR, whereas the intestinal β mRNA has only a 46-base 5′ UTR (Figure 2A). This extension of the human meprin β′ mRNA 5′ end could account for the size difference between the β and β′ mRNAs noted by northern blot analysis [21]. However, all the protein coding sequences in the β′ and β 5′ RACE products, including the signal peptide, propeptide, and portions of the protease domain, were identical.

The additional 5′ UTR sequence found in the human meprin β′ 5′ RACE product was shorter than the mouse meprin β′ 5′ UTR, which is 277 bases, and these two sequences had little similarity (25% base identity) (Figure 2B). The sequences encoding the mouse meprin β′ signal peptide and portions of the propeptide region also had little similarity to the human meprin β′ sequence. However, sequence comparisons of the human meprin β′ mRNA 5′ UTR to the mouse meprin β mRNA 5′ UTR revealed a great degree of similarity. When aligned at the translation start site, the 31-bp 5′ UTR of the mouse β mRNA was 71% identical with the human β′ mRNA (Figure 2C). The similarity of the human β′ and β sequence to the mouse β sequence extended throughout the signal peptide and propeptide coding regions as well.

Expression of Meprin β′ mRNA in other Human Cancer Cell Lines

The human meprin β′ mRNA isoform had previously been observed only in the human colon cancer cell line HT29-18C$_1$ [21] and had not been detected in the noncancerous kidney cell line 293 (Bond JS, unpublished results). To assess whether the meprin β′ mRNA is limited to colon cancer cells or is present in other human cancer cell lines, we examined meprin β′ mRNA expression in cancer cell lines derived from a number of different

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A.   HUMAN β'   ATTATTACAG CATCAAGCTG ACCTGCAATA TGGATCATTG AGATCAAAGG CTATAAATTA TAATGTTTGC CTTTAAAGAA    80
     HUMAN β'   GTCAATTCAA CCCTGAATGT CATAGTTAGC TACTTTCAAC TGGAAGCTAC AACATGGATT TATGGAATCT GTCTTGGTTT   160
                 ||| ||||||||||| ||||||||||| ||||||||||| ||||||||||| ||||||||||| ||||||||||| |||||||||||
     HUMAN β           CAA CCCTGAATGT CATAGTTAGC TACTTTCAAC TGGAAGCTAC AACATGGATT TATGGAATCT GTCTTGGTTT    73
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B.   MOUSE β'                                                                                  GACATGTG     8
     MOUSE β'   AGACGCTGGC TGGTCTCAAC AATTTGGCGA ACCAACGCCT GGGAAAGGCA GAGGACATTT GGAAGAAGCA CTGCTGGTTT    88
     MOUSE β'   GGAGCTCCAT GGAAGAAGAA AAGAATTAAA GAAAATTCGT ACCGGAGAAG ATACTGCTAC CTGCTGAGAG GCCTGTCCTG   168

     HUMAN β'   ATTATTACAG CATCAAGCTG ACCTGCAATA TGGATCATTG AGATCAAAGG CTATAAATTA TAATGTTTGC CTTTAAAGAA    80
                  |   | |  ||||||    | ||  |    | |     | | | ||   ||| |  ||  ||
     MOUSE β'   CACGTCATCA CCTGCAGCTG GTTCCAGGCT CCAAGGATGA ATCGGTGGGA ATGGACCTTC TCCCCTTTTC TTTATAAGGT   248

     HUMAN β'   GTCAATTCAA CCCTGAATGT CATAGTTAGC TACTTTCAAC TGGAAGCTAC AACATGGATT TATGGAATCT GTCTTGGTTT   160
                 | | | |   ||   | | | | |  |  || | |   |              |    | | |    |  |
     MOUSE β'   TTCCAGTTGA TGATGTTGCC TAGGGAGACA TGAATAGCAC AGCTGGACCA GCAAGCAGAA GCAGACACAG CTTCAAATGC   328

     HUMAN β'   CTGTTCTTGG ATGCTCTTCT CGTGATTTCT GGCTTGGCAA CTCCAGAAAA CTTT------ GATGTAGATGG CGGAATGGA   234
                  |  |        |    || || |     |             || |||||||| ||||| ||
     MOUSE β'   AGGATGAAGC TCCTGAAGGC TCCCAGGGAC GGGATGTACA TGATGACATT TGGAGTCAAA GACATAGATGG AGGAATTGA   408
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C.   HUMAN β'   ATTATTACAG CATCAAGCTG ACCTGCAATA TGGATCATTG AGATCAAAGG CTATAAATTA TAATGTTTGC CTTTAAAGAA    80
     HUMAN β'   GTCAATTCAA CCCTGAATGT CATAGTTAGC TACTTTCAAC TGGAAGCTAC AAC-ATGGAT TTATGGAATC TGTCTTGGTT   159
                 || | ||  ||||||| | ||||||||| || ||  |||||| || ||| ||| || | |||||||
     MOUSE β          AGCTTGC AGCTTTCATC TGGAAGCCAC AGTAATGGAT GCCCGGCATC AGCCTTGGTT    57
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Figure 2. Nucleotide sequence of the 5' end of human meprin β' mRNA isoform. The human meprin β' mRNA 5' end is compared to the previously determined sequence for human β cDNA (GenBank Accession # X81333) (A); the 5 end of the mouse meprin β' mRNA, which is expressed in teratocarcinoma cells (B); and the 5' end of the mouse meprin β mRNA expressed in normal kidney and intestinal cells

human tissues. (C). The caret indicates the location of the splice junction between the 3' exons common to both the mouse β and β' mRNAs (which is italicized) and the alternatively spliced 5' exons specific either to the mouse meprin β mRNA or to the mouse meprin β' mRNA. The translation start site (ATG) for each mRNA is underlined.

human tissues. To ensure that even low levels of the β' mRNA were detected, nested RT-PCR was used with primers specific to the β' mRNA 5' UTR sequence (obtained from the 5' RACE) and internal meprin β primers. The RT-PCR results revealed that the meprin β' mRNA was present in a number of other types of human cancer cell lines in addition to HT29-18C₁ (Figure 3), including the human breast cancer cell lines MCF-7 and SK-BR-3, the osteosarcoma cell line U2 Os, and the pancreatic cancer cell line BxPC-3. No meprin RT-PCR product was amplified from the human fetal kidney RNA with the β'-specific primers. Because previous work has shown that the human fetal kidney contains the meprin β mRNA [21], this confirms that the PCR primers were specific for the meprin β' sequences and that the β' and β mRNA isoforms are not coexpressed in fetal kidney cells.

PCR amplifications using primers for the constitutively expressed HPRT and GAPDH were used as controls to show that approximately equal amounts of template cDNA were present in each reaction. The levels of the PCR products for HPRT and GAPDH were equivalent in all samples (Figure 3). In addition, primers for the villin gene, which is expressed in brush-border membranes of kidney and intestinal epithelial cells, were used to show that a villin PCR product was found only from the intestinally derived HT29-18C₁ cell line and the human fetal kidney cell line RNA and not from the cell lines that do not contain brush-border membranes.

Putative Transcriptional Regulatory Elements in the MEP1B Genomic Upstream Region

Because the human meprin β' mRNA contains 87 additional bases at the 5' end compared with the β mRNA, the additional human β' sequences may be encoded by a separate exon or exons that are alternatively spliced in cancer cells. To identify the location of the 5' exons and promoter structure of the human MEP1B gene, the genomic region upstream of the meprin β gene, the genomic region upstream of the meprin β translation start site was cloned and sequenced. A 1.8-kb genomic clone, beginning within the translated region of the human MEP1B gene and extending upstream, was identified. Sequence analysis of the genomic region immediately upstream of the translation start site revealed that the 5' UTR sequences from the meprin β and β' mRNA isoforms were continuous in the genomic DNA (Figure 1). Thus, the arrangement of the human genomic DNA and mechanism used to produce the meprin β and β' mRNA isoforms in humans is different from the alternately spliced exon mechanism used to generate the mouse meprin β and β' mRNAs.

To characterize the human MEP1B promoter, approximately 700 bases of genomic DNA upstream of the meprin β subunit coding region was sequenced and compared with human promoter sequence databases. General transcription-factor binding sites, such as TATA boxes, were found 32 and 31 bases upstream of the β
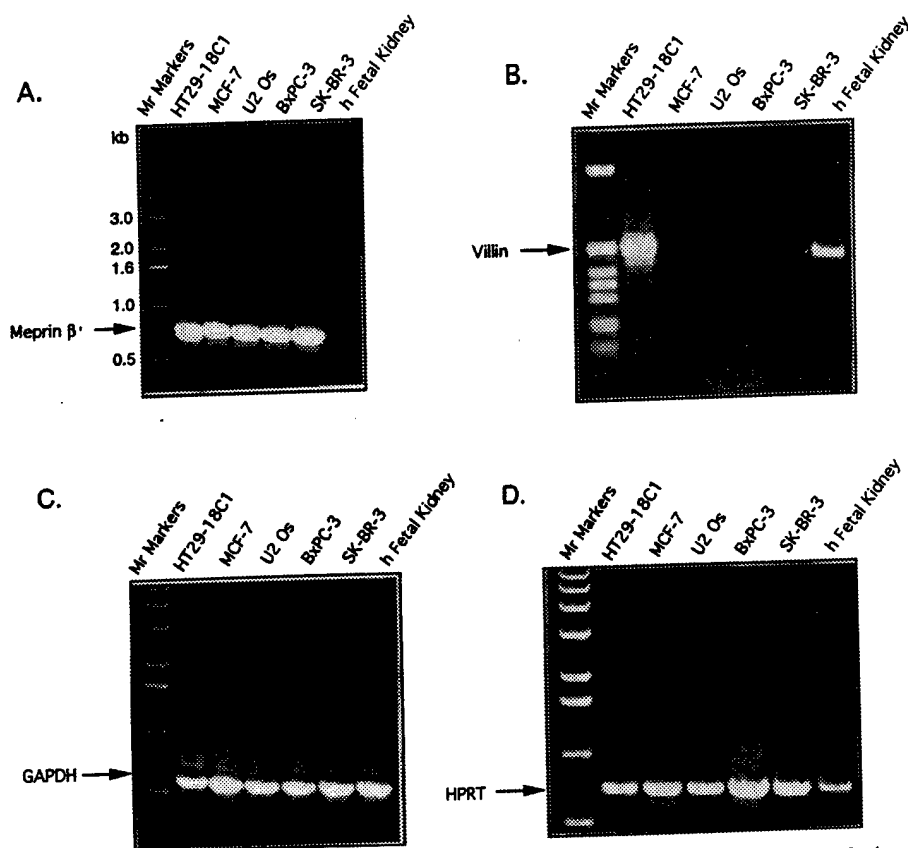
6

Figure 3. Expression of the human meprin β' mRNA in several cultured cancer cell lines. RT-PCR analysis of meprin β' mRNA (A), villin mRNA (B), GAPDH mRNA (C), and HPRT (A) mRNA levels in the cultured human cancer cell lines HT29-18C1 (colon adenocarci- noma), MCF-7 (breast carcinoma), U2 Os (osteosarcoma), BxPC-3 (pancreatic carcinoma), and SK-BR-3 (breast carcinoma), and in human fetal kidney (Fetal Kidney). The molecular weight (Mr) markers are a 1-kb DNA ladder (Life Technologies).

and β' transcription start sites, respectively. Several possible intestine-specific transcription-factor binding sites, which may be involved in regulation of transcription from the meprin β start site, were identified. Putative binding sites for the cdx-2 transcription factor, which regulates intestinal expression of the sucrase-isomaltase (SI), lactase phlorizin hydrolase (LPH), and proglucagon genes, were found at nt-157, -277, -296, -320, and -484 upstream of the β' transcription start site [23–25]. Three of these sites, nt-277, -296, and -320, were oriented in inverted fashion, as in the SI promoter, but were separated by 19 and 14 bases rather than the 2–3 bases separating these elements in the SI promoter [26]. A cdx-2 binding site similar to that found in the LPH promoter (LPH cdx-2, nt-157) was also found in the meprin β promoter. In addition, a putative binding site for the CCAAT/enhancer-binding protein (C/EBP β) transcription factor, which is expressed both in the intestine and kidney, was present at nt-606 [27]. Kidney-specific promoter elements were less well-defined, but a poten- tial intermediate/weaker affinity nkx 2.5 binding site, which is generally associated with cardiac-specific genes, was detected at nt-91, and a higher-affinity nkx 2.5 binding site was detected at nt-167 [28]. The kidney- restricted type II promoter for the mouse γ-glutamyl

transpeptidase (GGT) gene also contains potential nkx 2.5 and C/EBP β transcription-factor binding sites [29].

Also within the meprin β promoter region were several areas of sequence homology to elements known to affect gene expression in cancer cells (Figure 1). These include a putative AP-1 binding site at nt-450, a putative PEA3 site at nt-607, and an overlapping AP-1/PEA3 site at nt-82. Both of the putative PEA3 elements conformed to a consensus sequence (AGGAAA), whereas the putative AP-1 elements each differed from consensus by 1 base (TGACCCA and TGACACA). A search for other areas of AP-1/PEA3 pairing within the MEP1B promoter did not reveal other sites with similar element proximity. Also within this region is a putative osteocalcin-specific element (OSE2) motif (nt-59) with similarity to elements in the osteocalcin and MMP-13 promoters [30–33]. Finally, putative half-consensus estrogen-responsive elements (1/2 EREs) were found at nt-450, -468, and -684.

Transcription from the MEP1B Promoter in Cancer Cells

To examine the roles of the putative transcription- factor binding sites, U2 Os cells were transiently trans- fected with reporter-gene constructs containing a luciferase gene and several 5' deletions of the MEP1B
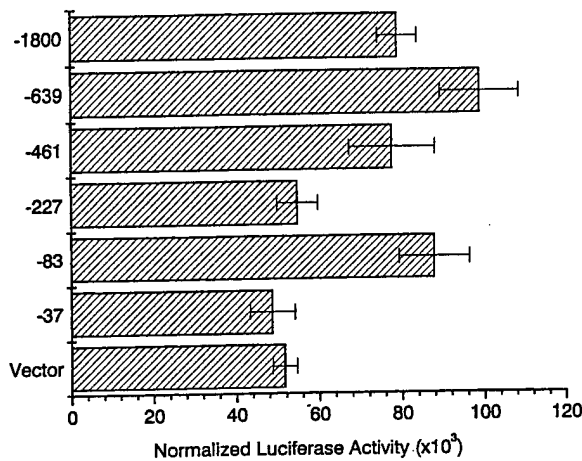
Figure 4. Luciferase reporter gene activity, normalized to β-galactosidase activity, of the human *MEP1B* promoter constructs transiently transfected into U2 Os osteosarcoma cells. "Vector" represents the pGL3-enhancer vector alone whereas "-37", "-83", "-227", "-461", "-639", and "-1800" represent *MEP1B* genomic sequences upstream of the human meprin β' transcription start site (as determined by 5' RACE). The error bars represent the standard error of the mean of at least 12 independent transfections.

promoter. U2 Os transfections with a minimal TATA-containing promoter fragment (nt-37) gave reporter-gene activity that was indistinguishable from that of the promoterless vector control (Figure 4). A construct containing promoter sequences from nt-37 to -83 produced an increased level of luciferase activity, and,

with the exception of the -227 promoter construct, the addition of more promoter sequences (up to 1.8 kb of genomic DNA) produced similar levels of luciferase activity. The luciferase activity generated by the -227 promoter construct was not different from those of the -37 construct or vector-only control. As an additional control, the -1800 construct was also tested for its expression in a nontransformed cultured cell line, 293 human embryonic kidney cells, which do not express meprin β' or β mRNA. In five independent transient transfections, no promoter activity was detected in this cell line (data not shown).

Phorbol Ester–Induced Meprin β' mRNA in Colon Cancer Cells

The presence of AP-1 and PEA3 sites in the *MEP1B* upstream region indicated that expression of the human meprin β' mRNA could be affected by tumor promoters such as phorbol esters. Phorbol esters increase transcription of genes encoding several other proteolytic enzymes, and the promoters for these genes contain similar AP-1 and PEA3 elements [11]. After a 24 h incubation in serum-free medium, the human colon cancer cells (HT29-18C$_1$) contained nearly undetectable levels of meprin β' mRNA, as determined by RT-PCT. Treatment with 50 or 100 ng/mL of the phorbol ester PMA, which induces MMP-7 (matrilysin) expression in the human colon cancer cell line SW620, resulted in a marked increase in the amount of meprin β' mRNA (Figure 5). The levels of the
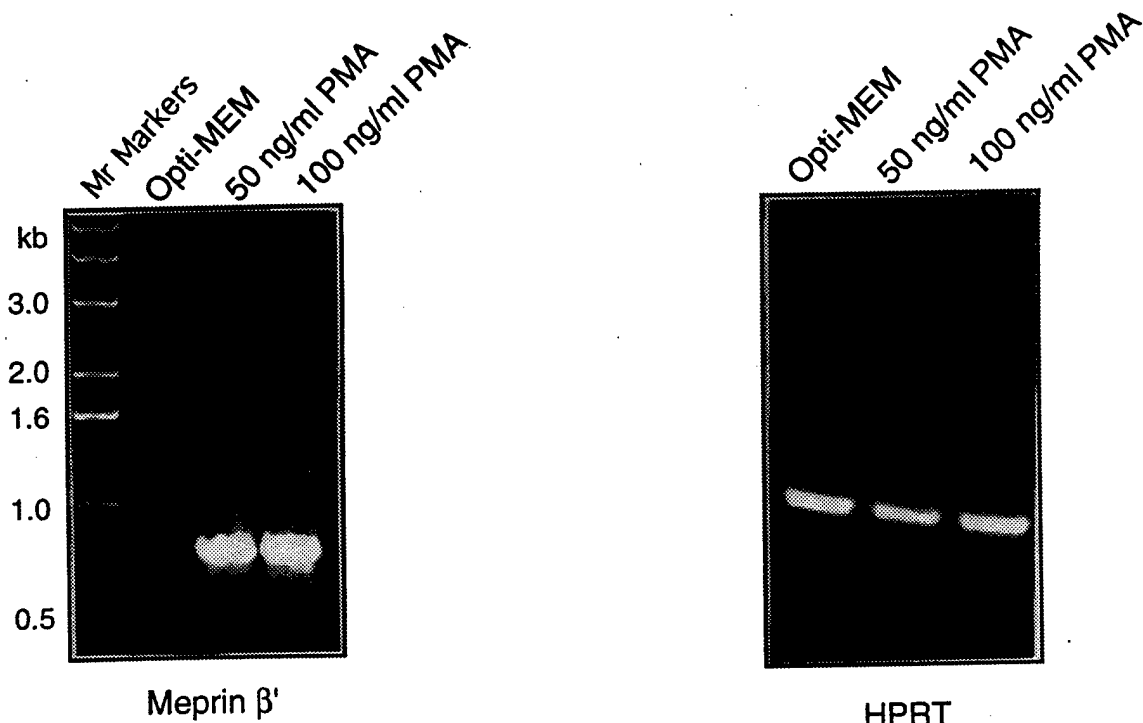

Meprin β'

HPRT

Figure 5. Induction of the human meprin β' mRNA by the phorbol ester PMA. Cultures of the human colon adenocarcinoma cell line HT29-18C$_1$ were transferred to serum-free medium (Opti-MEM) overnight and then treated with 50 or 100 ng/mL PMA for 8 h. After harvesting of the cells, RNA was extracted, reverse transcribed, and used for PCR with either meprin β'-specific 5' primers or HPRT primers (as a control).

constitutively expressed HPRT mRNA were unaffected by PMA treatment.

Phorbol-Ester-Induced Increase in the Activity of the *MEP1B* Promoter/Luciferase Constructs

The role of specific AP-1 or PEA3 elements in the phorbol-ester induction of meprin β' mRNA was further explored by PMA treatment of U2 Os cells transfected with the *MEP1B* promoter/luciferase constructs. The segment of the *MEP1B* promoter with the highest activity in untreated cells (-83), as well as the -461 promoter segment, did not show increased activity with PMA treatment (Figure 6). However, the construct containing -639 base pairs of the *MEP1B* promoter did show significantly increased levels of reporter gene activity with PMA treatment ($P = 0.07$), and the -1800 construct also had significantly higher activity ($P = 0.05$) with PMA treatment.

DISCUSSION

This study defined the differences between the human meprin β and β' mRNAs, established that the expression of the human meprin β' mRNA was not limited to colon cancer cells, and identified upstream genomic DNA sequences that are responsible for the unique expression of the human meprin β' mRNA in cancer cells.

The difference in size of the human meprin β and β' mRNAs lay in the sequence of the 5' end of the mRNA, as was found for the mouse meprin β' mRNA. However, unlike the mouse, meprins, the human meprin β and β' mRNAs were identical throughout the coding regions, containing the same propeptide, signal peptide, and 5' UTR sequence. Although the human meprin β' RACE product did contained an additional 5' sequence, this sequence has no similarity to the mouse meprin β' 5' UTR. In fact, the human meprin β' and β sequences shared a high degree of similarity to the 5' UTR, signal peptide, and propeptide of the mouse β mRNA isoform. The difference between the human β' and mouse β' 5' RACE products indicated that the meprin β' mouse and human mRNAs were generated in a different manners.

Two distinct mechanisms for producing the human and mouse meprin β' mRNAs were also indicated by the sequence of the human genomic DNA encoding the 5' end of the meprin β' transcript. The sequences encoding the human meprin β and β' mRNA 5' UTRs were contiguous in human genomic DNA. This differs from the genomic organization of the mouse *MEP1B* gene, in which the 5' UTR and NH$_2$-terminal protein-coding sequences (signal peptide and portions of the propeptide) for the β and β' mRNAs were present on separate exons [21] (Jiang, et al., manuscript in preparation). The arrangement of the human gene implies that the meprin β and β' mRNAs share a single promoter with multiple cis-acting elements that directs meprin expression from different transcription start sites in cancer cells and in normal tissues. A similar, single mixed promoter is found upstream of another human protease gene, cathepsin D [31]. The cathepsin D gene contains a single promoter that directs transcription from multiple start sites in both normal cells and in cancer cells. The cathepsin D promoter includes Spl elements that direct constitutive expression of the lysosomal form of the protease as well as estrogen-responsive elements and other promoter sequences that direct expression of an extracellular form of the enzyme in breast cancer cells [31].

The potential for a mixed promoter containing elements to direct transcription of both the human meprin β and β' mRNAs is further indicated by the presence of several potential cdx-2 binding sites in the region upstream of the meprin β transcription start site, as are found in the promoters of other intestine-specific genes. These genomic elements could direct the intestinal expression of the meprin β mRNA. Further upstream, both the meprin β and GGT promoters contain consensus binding sites for the intestine- and kidney-expressed transcription factor C/EBP β, which also may be involved in the tissue-specific expression of these genes. In contrast to the *SI* promoter, the meprin promoter did not contain an HNF-1 binding site, which also regulates intestine-specific transcription of the *SI* gene [32].

Identification of promoter elements that could direct meprin β expression in the kidney proximal tubules is more difficult, because of the scarcity of information on kidney-specific promoter sequences. The kidney proximal tubule—specific mouse GGT type II promoter does contain putative AP-1, C/EBP β, and nkx 2.5 binding sites, as does the meprin β promoter. The meprin β promoter does not, however, contain AP-2 or GRE sites, which influence transcription of the mouse GGT Type II promoter [29].

Functional analysis of the putative promoter elements directing meprin β' expression in cancer cells revealed that the meprin β' promoter contained both positive and negative regulatory elements. Based on reporter-gene activities of the promoter constructs in cancer cells, the promoter segment from nt-37 to -83 contains sequences critical for transcription in U2 Os cells. However, the reporter gene activity of the -277 construct, which contains both of the nkx 2.5 binding sites, was equal to that of the vector alone, indicating that the region from nt-227 to -83 contained negative regulatory elements. The COOH-terminal portion of the nkx 2.5 homeodomain protein can act as a transcriptional repressor [28] and may account for the low activity of the -227 construct. The presence of putative AP-1 and PEA3 elements may account for the expression of the meprin β' in cancer cells. Promoters from several other proteinases, including MMP-1 (interstitial collagenase), MMP-3 (stromelysin 1), MMP-7 (matrilysin), MMP-10 (stromelysin 2), MMP-13 (collagenase 3), and the urokinase plasminogen activator also contain both AP-1 and PEA3 elements [9, 34]. The location and orientation of these elements with respect to the transcription start site and with respect to each other varies, but most contain one or more upstream PEA3 sites. Phorbol esters, such as PMA, act through increasing levels of the transcription factors that bind to AP1 and PEA3 sites [13]. Thus, genes whose promoters
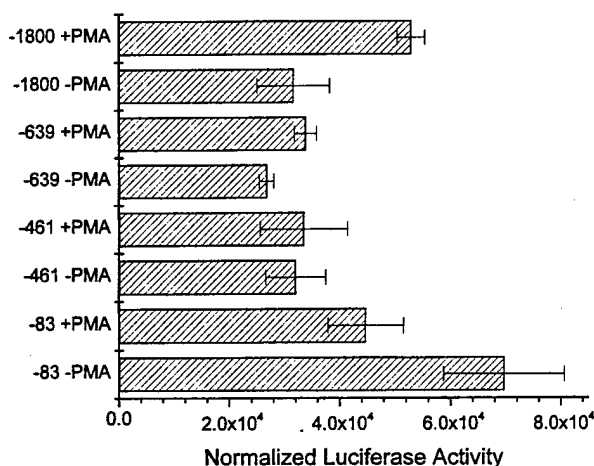
Figure 6. Changes in *MEP1B* promoter/luciferase reporter gene activity in response to the phorbol ester PMA. The *MEP1B* promoter DNA/luciferase constructs were transiently transfected into U2 Os cells. After 24 h, the cells were treated with 100 ng/mL PMA, and after an additional 24 h, the luciferase activity was measured. The error bars represent the standard error of the mean of seven or eight independent transfections.

contain these transcription-factor binding sites should show increased levels of mRNA in response to PMA treatment. Thus is true for the meprin β' mRNA in the colon cancer cell line HT29-18C₁ (Figure 5). Placing HT29-18C₁ cells in serum-free medium overnight decreased the levels of meprin β' mRNA expression, presumably because of the lack of growth factor or other serum component required for optimal meprin β' expression, but the enhanced expression of meprin β' mRNA in response to phorbol ester treatment indicates that the AP-1 and PEA3 elements in the meprin β' promoter functioned as would be predicted. Further examination of the activity of the reporter gene constructs in U2 Os cells, both with and without PMA treatment, indicated that the segment of the *MEP1B* promoter upstream of nt-461 was the most critical to phorbol ester induction of meprin expression in cancer cells (Figure 6). The presence of a PEA3 element in this region of the promoter could be critical for the phorbol ester induction of reporter-gene activity. Because the -83 construct did not show PMA inducibility, the OSE2 element in this region may be more important to the activity of this promoter region in the U2 Os osteosarcoma cells than the overlapping AP-1/PEA3 element at nt-82. AP-1 and PEA3 sites within the *MEP1B* promoter may also interact with other elements, such as the three putative 1/2 EREs. Tandem copies of the 1/2 ERE elements also are found in the cathepsin D promoter, which is hightly expressed in breast cancer cells [33]. Because meprin β' expression was detected in two human breast cancer cell lines, MCF-7 and SK-BR-3, further investigation of the β' promoter constructs in estrogen receptor–positive mammary cancer cell lines is warranted.

The studies reported here indicated that the mechanisms of meprin β' and meprin β expression in human and mouse cancer cells are distinct. Human meprin β and meprin β' mRNAs produce identical proteins, whereas mouse meprin β' and β mRNAs produce proteins with different N-terminal sequences [21]. It is possible that the DNA sequences homologous to the mouse β' exons exist upstream of the human *MEP1B* gene but that these exons are not used in either human cancer cells or in normal epithelium. The DNA sequence of the splice junction where the mouse β' exons are joined to the common exons is different in the human *MEP1B* gene. There is a 6-bp deletion in the human gene compared with the mouse gene (Figure 2). This deletion could result in a splice junction that can no longer accept the potential β' upstream exons.

Why make two mRNAs that differ only in their 5' UTRs. One possibility is that the meprin β' and β mRNAs are translated with different efficiencies or have different stabilities. Another possiblity is that by using different promoter elements to direct transcription from different start sites, meprin β' and β mRNAs can be independently regulated for the amount of mRNA produced, the timing or tissue specificity of expression, or the type of factors that induce their expression.

Recombinant rat meprin β protein has been shown to cleave the regulatory subunit of protein kinase A and the peptide hormore gastrin *in vitro* [35, 36]. This suggests that the human meprin β protein subunit has a role in regulating the levels of peptide hormones or the activity of regulatory proteins such as protein kinases in cancer cells. Because human meprin β' mRNA contains an intact signal peptide and a transmembrane domain, which positions the protein in the plasma membrane, the bulk of the meprin β subunit is probably extracellular on cultured human cancer cells, as on intestinal brush-border membranes. This places the meprin β protein in a critical location, at the surface of cancer cells, where it may affect levels of peptide hormones, activate other latent proteases or growth factors, or regulate the activity of other proteins by limited proteolysis.

ACKNOWLEDGEMENTS

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